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בקשה לפטנט
Application for Patent

אני, (שם המבקש, מעט ולגבי גוף מאגד - מקום התאגדות)
(Name and address of applicant, and in case of body corporate-place of incorporation)

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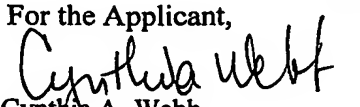
מכשיר להדמיה פלואורוסנטית של דוגמאות ביולוגיות תוך שמוש במיקרוסקופ
אלקטרוני סורק יחד עם סמנים פלואורוסנטיים או סמני סינטלציה

(באנגלית)
(English)

Device for Fluorescent Imaging of Biological Samples using a Scanning
Electron Microscope and Fluorescent or Scintillation Markers

hereby apply for a patent to be granted to me in respect thereof.

מבקש בזאת כי ינתן לי עליה פטנט

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For the Applicant,  Cynthia A. Webb Patent Attorney						
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**Device for fluorescent imaging of biological samples using a scanning electron
microscope and fluorescence or scintillation markers**

**מכשיר להדמיה פלואורסצנטית של דוגמאות ביולוגיות תוך שימוש במיקרוסקופ
אלקטרוני סורק יחד עם סמנים פלואורסצנטיים או סמני סנטילציה**

QMX/009

Device for fluorescent imaging of biological samples using a scanning electron microscope and fluorescence or scintillation markers

Field of the invention

5 The invention relates to a device that allows high resolution imaging of biological cells or any other sample using an electron microscope and scintillation or fluorescence markers. More specifically, the invention comprises an apparatus that allows the measurement of light emitted from the sample due to the excitation by the fast electrons of a scanning electron microscope (SEM) in conjunction with a unique
10 labeling technique using nanoscale scintillating spheres.

Background of the invention

High resolution imaging of wet samples is of great value to many fields. Including life science research, agriculture, food industry and materials sciences. In the
15 life sciences, information such as protein translocation, distribution of membrane proteins and distribution of proteins in relation to specific organelles, is important to the understanding of cellular structure and processes and to assay drug action. In many applications, fluorescence microscopy can yield an informative readout. However, light microscopy has some limitations.

20 The best attainable resolution of Light Microscopy (LM) is in the range of several hundreds of nanometers. Furthermore, the signal to noise ratio also limits the sensitivity of detection. Among the advantages of LM for life sciences is the ability to image fluorescent markers, which are compatible with the biological samples and allow multi color labeling.

Conventional Electron Microscopes (EM), on the other hand, achieve very high signal to noise ratios and a very high resolution. Commercially available Scanning Electron Microscopes (SEMs) can reach a resolution of one nanometer or less. However, they suffer from major limitations that have so far limited their utilization, particularly in life science research. The fact that electrons require vacuum in the path of the beam means that biological samples cannot be observed in their native wet environment. Consequently, the conventional electron microscope cannot be used to image living cells. Even when dealing with fixed cells, conventional EMs require tedious sample preparation procedures that distort important biological information. These sample preparation technologies impeded the automation of electron microscopes for life sciences use. Therefore, electron microscopes cannot currently serve as an efficient drug screening tool.

Co-pending International Patent Application Serial No. PCT/IL01/01108 by one of the present inventors, discloses a chamber adapted for scanning electron microscopy in a wet environment, the content of that application being incorporated herein in its entirety. PCT/IL01/01108 discloses a chamber adapted for use with a scanning electron microscope, wherein the chamber has at least one aperture sealed with a membrane, which is adapted to withstand a vacuum, and is transparent to electrons. By this configuration, the chamber is isolated from the vacuum, enabling the scanning of wet samples. Though that application further provided certain embodiments for working with fluorescent labelling as well as scanning electron microscopy in a wet environment, the solutions provided therein were not suitable for working with low signal levels. The present invention overcomes certain limitations of the previously disclosed system.

Processes of light emission due to the electron beam impact, sometimes referred to as cathodoluminescence (CL), have been studied and used for many years in different fields such as geology, semiconductors, material science and more. Extensive research was performed on dehydrated biological samples in the 1970's and 1980's, however, these studies were limited by the effects of dehydration of the samples and the weakness of the signal measured (Paul V. C. Hough, 'Cathodoluminescence – detailed understanding may yield in situ distributions of individual molecular species at 100Å resolution or below', Scanning Electron Microscopy 1, 258 (1977); W. Brocker and G. Pfefferkorn, 'Application of the cathodoluminescence method in biology and medicine', Scanning Electron Microscopy, 2, 125 (1979)).

Summary of the Invention

The current invention discloses a device enabling Electron Beam Fluorescence (EBF) in wet samples, opening revolutionary possibilities for analysis of cellular function and of drug action. The present invention discloses the utilization of existing fluorescent labels for imaging cells by means of EBF as well as providing novel fluorescent labels for imaging cells at high resolution. The present invention thus provides higher collection efficiency of EBF compared to that achieved by the apparatus and methods of PCT/IL01/01108, thus enabling high resolution fluorescence imaging of a sample maintained in a wet environment.

This system is easy to automate since the sample preparation steps are as easy as with LM. The current invention expands the technology of SEM in a wet environment and allows the extraction of optical signals from live and wet biological samples with resolution of tens of nanometers. This introduces a realm of new possibilities unattainable by other methods.

The combination of samples maintained in a wet environment together with readout of fluorescent signals, as disclosed in the current invention affords electron microscopy the versatility of optical imaging with fluorescent markers, which is now a major technological tool in molecular and cell biology. The central idea in the current invention is to use the electron beam to excite target compounds in the sample and then measure the light emitted from it. The main advantage of this technique over standard optical imaging is the ability to perform localized excitation by the electron beam (rather than excitation by photons which are not as localized) and hence achieve a resolution which is of the size of the excitation region. This size can be only a few nanometers in the case of an electron microscope (depending on the different settings of the SEM).

The current invention combines the advantages of both EM and LM. It is based on an electron microscope that can visualize wet samples, using the diversity of convenient labels that were developed for LM and in parallel to the readout of the electrons signal such as back scattered electrons, thus allowing the user additional information.

A crucial issue in this technique is the ability to find a suitable labeling agent. Numerous materials and chemicals are known to fluoresce under electron radiation. Many standard fluorophores are known or expected to have some CL capabilities (Paul V. C. Hough, 'Cathodoluminescence — detailed understanding may yield in situ distributions of individual molecular species at 100Å resolution or below', Scanning Electron Microscopy 1, 258 (1977); W. Brocker and G. Pfefferkorn, 'Application of the cathodoluminescence method in biology and medicine', Scanning Electron Microscopy, 2, 125 (1979); M. De Mets and A. Lagasse, 'An investigation of some organic chemicals as cathodoluminescent dyes using scanning electron microscope', Journal of

Microscopy, 94, 151 (1971)). Under wet conditions many other standard fluorophores or even fluorescent proteins can serve as good labeling candidates. Common problems with such materials are weak signals and rapid bleaching (see for example in Paul V. C. Hough, ' Cathodoluminescence – detailed understanding may yield in situ distributions of individual molecular species at 100Å resolution or below', Scanning Electron Microscopy 1, 258 (1977). We propose here to solve these problems by utilizing nanometer scale spheres made out of scintillating materials (for a review see the book by J. B. Birks, "The theory and practice of scintillation counting", Pergamon Press, 1964). Scintillating materials are materials that have high photon emission efficiencies, namely, many photons for each electron hitting the material. Scintillating materials are commonly used for radiation detection specifically in detectors for electron microscopy and radioactivity. Small nanoscale beads made out of such materials can produce a much higher signal than standard fluorophores and are also less prone to bleaching by the electron beam. Such scintillating beads can be made from standard scintillating materials such as scintillating plastic or from glassy scintillating materials.

It should be noted that similar scintillating beads have been used in a different application of scintillation proximity assay (SPA) where 5µm size scintillating beads attached to antibodies are used as radioactivity sensors placed in close proximity to radioactive markers.

20

Brief description of the Figures

Figure 1 presents drawings of a sample chamber for SEM combined with EBF, and its individual component parts. Figure 1A: presents a generalized longitudinal cross section of a Sample Chamber for EBF; Figure 1B: drawing of the cavity disk; Figure 1C

drawing of the frame; Figure 1D: Light guide with conical edge; Figure 1E: Housing for EBF chamber; Figure 1E: support for EBF chamber.

5 **Figure 2** presents drawings of an EBF chamber with horizontal light collection system; Figure 2a: Horizontal light guide. Figure 2b: Flange for the horizontal light guide; Figure 2c: Outer flange for horizontal light guide; Figure 2d: Protective shield for horizontal light guide; Figure 2e: Stage plate for the EBF setup.

Figure 3: Left panel: Imaging of a wet HeLa cell with electron beam fluorescence. Right panel: Simultaneous image taken with back scattering detector. Scale bars of the left picture is as shown on the bottom of the right picture.

10 **Figure 4:** Fluorescent beads immersed in water imaged using the electron beam fluorescent setup at different magnification. Left panels: optical imaging. Right panels: simultaneous back scattering imaging. Scale bars of left pictures are as shown on the bottom of their corresponding right pictures

15 **Detailed description of the invention**

The present invention provides apparatus for the simultaneous back scattering electron microscopic imaging of samples particularly biological cells, while collecting electron beam fluorescence from the same samples.

The invention consists of two main advances over previously known devices:

20 1. A sample chamber and light collection system that allows imaging of biological cells or any other wet sample in the SEM. This is performed by combining a light collection and detection system with the principles of a sample chamber designed for scanning electron microscopy of samples in a wet environment.

2. A labeling technique utilizing nanometer scale scintillating beads that bind to specific binding sites on the imaged cells, allowing high resolution, multi color imaging.

5 Sample chamber and light collection and detection system

The sample chamber is based on the chamber disclosed in a previous patent application PCT/IL01/01108, by one of the present inventors and others.

Co-pending International application PCT/IL01/01108, entitled "Device and method for the examination of samples in a non-vacuum environment using a scanning
10 electron microscope"), the content of which is hereby incorporated by reference, discloses a non-vacuum SEM device that allows imaging cells with SEM in a wet environment. This is accomplished with the use of a thin membrane, also termed hereinafter a partition membrane, that is transparent for electrons but is strong enough to withstand the pressure difference between the inside of the chamber and the sample
15 space vacuum. In general, the chamber is located in a vacuum whilst enclosing a sample or samples within a fluid, or at substantially atmospheric pressure, or both.

In accordance with one particular embodiment of the present invention (Figure 1) the sample chamber comprises the following components parts:
a cavity disc (4) having a top surface and a bottom surface wherein the top surface
20 of the disc is sealed by a partition membrane (7);
the partition membrane (7) sealing the top of the cavity disc having an outer surface and an inner surface, said inner surface facing the cavity of said cavity disc, said partition membrane being transparent to electrons;
optionally a grid affixed to the outer surface of the partition membrane;

a frame element (3) for holding the cavity disc (4), the frame element having an aperture extending at least over the width of the cavity;

a housing (1) enclosing the framed cavity disc and a support element (2) having attachment means (6) for attaching the frame to the housing and its support;

5 a light guide (5) positioned to collect emitted light from the sample.

In principle, the sample chamber has a thin partition membrane (7) on the inner surface of which the cells are situated or cultured and it remains wet during imaging. While the electron beam is scanning this partition membrane from the top or outer side,
10 light is collected through a light guide (5) positioned just below the sample said sample situated on the partition membrane.

According to the present invention the light guide advantageously has a conical shaped edge (8) to improve light collection efficiency. This setup allows simultaneous measurement of both the back-scattered electrons and the fluorescence signal from the
15 sample since the light collection system does not interfere with the collection of back-scattered electron, thus providing an advantage over commercial cathodoluminescence systems. This setup also allows an efficient collection of the light since the light guide is collecting a large fraction of the photons emitted. The light collection is enhanced by the relatively good matching between the indices of refraction of the medium the
20 sample is immersed in and the material of the light guide (glass, plastic, etc.). Note that the light guide does not interfere with sample preparation and all the advantages of imaging using the wet environment sample chamber technology are applicable to this system as well.

Another embodiment according to the invention (Figure 2) provides a horizontal light detection system. The light emitted from the sample is first collected through the light guide (5 in Fig 1A) of the sample chamber, then light is transferred to a horizontal light guide (5 in Figure 2A) going through the wall of the microscope chamber, and finally brought into a photo multiplier tube detector (12) (PMT). Figures 2b-f show the detailed components of the horizontal light collection system.

According to the present invention the horizontal light guide is made out of polished Perspex or any other optical grade plastic or glass. The half cone shaped edge (15) below the sample chamber is coated by reflecting layer such as aluminum or gold, and is designed to reflect all the light coming from the sample chamber light guide into the horizontal light guide (5 in Figure 2A). The position of this light guide can be manipulated via three plastic screws (16) in the aluminum holder (17) (Figure 2C). The aluminum holder (17) is further tightened by screws (13) to the protective cover (14) of the horizontal light guide. The light guide extends through a vacuum tight feedthrough at the wall of the microscope chamber.

The PMT (12) is placed right at the outer edge of the horizontal light guide. The PMT used is a high sensitivity photon counting detector such as is made for example by Hamamatsu. Filters can be placed in front of the PMT in order to select a specific wavelength detected.

The signal from the PMT could then be read via a computer controlled counter card, which is synchronized with the scanning rate of the SEM through dedicated software written for this purpose. The images of the EBF and the back scattering image are displayed simultaneously on two separate screens allowing direct comparison of the two signals.

Alternative light collection and detection are also possible including placing the PMT inside the microscope vacuum chamber, using flexible optical fibers instead of light guides, collecting light from the top side of the sample as well, or introducing a spectrometer in front of the PMT to allow spectral analysis of the optical signal obtained.

Sample imaging and labeling

The ability to image biological cells is shown in Fig. 3, where unlabeled HeLa cells are imaged using the setup described above. This demonstrates that biological material can also produce autofluorescent signal under electron impact. Even though unlabeled cells can also be imaged, more detailed and specific information can be obtained by using specific labeling. Labeling in such a setup can be fluorescent chemicals or fluorescent proteins that can be excited by the electron beam. A large variety of such fluorophores and fluorescent proteins were developed in the last couple of decades for fluorescent microscopy. It is likely that many of these fluorophores can be applied to this technique.

The disadvantage of chemical fluorophores is that the emission of light is rather weak and that some of these materials can bleach very fast with an exposure to an electron beam (Paul V. C. Hough, 'Cathodoluminescence – detailed understanding may yield in situ distributions of individual molecular species at 100Å resolution or below', Scanning Electron Microscopy 1, 258 (1977), W. Brocker and G. Pfefferkorn, 'Application of the cathodoluminescence method in biology and medicine', Scanning

Electron Microscopy, 2,125 (1979)). To resolve these problems we suggest a unique labeling technique of nanoscale scintillating beads. The basic idea is to take a scintillating material and make small nanoscale beads from it. Such beads can then be attached to specific sites on the samples through protein-ligand interactions such as antibody-antigen, biotin-avidin or sugar-lectin. This technique has the following properties:

1. High signal – such scintillating beads have high emission of photons and hence a high signal is expected even for small region scanned.
2. High resolution – the ideal size of the beads should be on the range of tens of nanometers. This should allow highly specific binding up to the single molecule scale. The small scale also reduces any adhesion problems, due to steric configuration of the labeled sample.
3. Low bleaching – Standard solid scintillating materials undergo only weak bleaching under electron beam irradiation, hence such beads are expected to have persistent signal during the imaging time.
4. Multicolor – such scintillating beads can be designed to emit at different wavelengths. Such ability can allow straightforward application to multi labeling where different molecules are labeled at different colors. This is one of the main advantages of these labels over standard colloidal gold labeling.
5. Co-labeling with electron microscopy labeling – since image can be obtained simultaneously for the optical imaging and back scattering imaging, co-labeling with gold colloids or other electron microscopy labels can be achieved. This increases the possibilities of the system.

A central issue here is the ability to produce such beads with size range of tens of nanometers using scintillating materials. In order to make such beads plastic scintillating materials like NE102 (sold as BC400 by Bicron) or glassy scintillating materials such as Yttrium silicate doped with Ce can be used (sold as P47). Beads with the sizes required can be produced from these materials by standard techniques. As an example, similar beads, optimized for fluorescence microscopy, which are made from polystyrene with sizes down to 20nm are available in the market (Fluospheres® by Molecular Probes). Figure 4 shows how 1µm fluorescent beads are imaged with high resolution using the imaging setup described above. In order to attach the beads to specific binding sites they should be coated with a labeling reagent such as specific antibodies, protein A, Biotin or Avidin, etc. Such coating is also a standard procedure for plastic or glass beads.

A major advantage and a powerful tool in cellular biology and drug discovery research could be the use of an EBF protein. These proteins could be expressed under various control sequences in living cells in culture under normal conditions and imaged for BSE and EBF as live cells in normal physiological environment. The first obvious candidate is the green fluorescent protein (GFP) and variants, which include the EGFP, EYFP, ECFP, GFPuv, hrGFP and others. These proteins have proven their robust capabilities as fluorescent protein markers in live and fixed cells. Unfortunately, neither over-expressed nor purified protein of the GFP variants had any measurable EBF. For the development of new suitable EBF markers several approaches are being taken in order to produce a marker that has the desired properties to enable its use for EBF.

EXAMPLES

Assembly Of The EBF Chamber And Light Detection And Collection System.

5 A schematic drawing of the EBF chamber is shown in Fig. 1. The main parts to the chamber:

- a. Cavity disk (See Fig. 1B) – a Perspex disk with a Polyimide membrane and a Nickel grid glued to its top surface. The cavity disk is where the cells are cultured and labelled.
- 10 b. Frame (See Fig. 1C) – an aluminum plate in which the cavity disk is situated.
- c. Light guide with conical edge (See Fig. 1D) – made from coated glass.
- d. Housing (See Fig. 1E) – the first part for closing the chamber,
15 made out of Ertalyte (plastic).
- e. Support (See Fig. 1F) – the second part for closing the chamber.

To prepare an EBF chamber for viewing in the SEM the following steps are performed:

20

- a. Place a cavity disk inside a frame with the polyimide membrane facing downward. All handling from now on will be performed with the frame and the cavity disk together.

- b. Place an O-ring (9) around the light guide and close it between the housing and the support with three screws (6). The light guide (5) should be positioned so that its conical edge (8) is close to the position of the membrane (7).
- c. Place another O-ring (9) inside the housing and when the sample is ready close the chamber with three screws (6).
- d. To check that samples are vacuum tight, place them inside a vacuum chamber for a few minutes (until pressure $< 10^{-2}$ mbar).

In order to set up the light collection and detection system (Fig. 2A) the following stages are performed:

- a. Put the horizontal light guide (5) (Fig. 2B) inside the flange (17) (Fig. 2C) and fasten the light guide into position using an O-ring (9) and the outer flange (18) (Fig. 2D). The position of the horizontal light guide should be such that its flat edge is centered below the beam position.
- b. Take the flange with the horizontal light guide attached to it and place it on the suitable opening in the microscope's vacuum chamber. The proper O-ring (9) should be placed in order to seal the vacuum chamber. The flange drawn in Fig. 2C is designed to fit a commercial XL30 SEM made by FEI.
- c. The exact position of the horizontal light guide can be controlled using plastic screws (16 in Fig. 2A).
- d. The protective cover (14) (Fig. 2E) should be placed on top of the light guide after final position is set.
- e. Connect the stage plate for the EBF (11) (Fig. 2F) and place the EBF chamber in one of the holes in the plate. Set the position of the stage so that the EBF

chamber is below the electron beam and above the flat part (15) of horizontal light guide. It is advisable that the two light guides are positioned very close to each other in order to achieve best light collection efficiency.

- 5 f. A PMT (12) can now be attached to the outer flange using an adaptor flange. A filter can be placed in front of the PMT if needed.

Images of samples including viable cells obtained with the chambers of the invention

10 Figures 3 and 4 present images obtained using the chambers of the invention, including biological samples that can be viewed as SEM electron back scattering images and electron beam fluorescent images simultaneously.

Figure 3: Left panel shows imaging of a wet HeLa cell with electron beam fluorescence. Right panel shows simultaneous image taken with back scattering detector. Scale bars of the left picture is as shown on the bottom of the right picture.

15 Figure 4: Fluorescent beads immersed in water imaged using the electron beam fluorescent setup at different magnifications. Left panels show optical imaging. Right panels show simultaneous back scattering imaging. Scale bars of left pictures are as shown on the bottom of their corresponding right pictures

CLAIMS

1. An apparatus for high resolution inspection of an object in a chamber using a scanning electron beam, comprising a vacuum path for the electron beam and a vacuum-resistant partition membrane placed in association with the object, said vacuum resistant partition membrane being placed such as to isolate the object from the vacuum, wherein the object comprises one of a group comprising a scintillating bead, a fluorescent protein capable of electron beam fluorescence and an electro-luminescent marker, whereby a beam of electrons are able to excite photo-emission.
5
- 10 2. The apparatus according to claim 1, wherein said chamber comprises part of a light guide.
3. The apparatus of claim 2 wherein the light guide has a conical shaped edge to
15 improve light collection efficiency.
4. The apparatus according to claim 1, further comprising a horizontal optical sensing unit.
- 20 5. The apparatus according to claim 4, wherein said optical sensing unit comprises a photo-multiplier tube.
6. The apparatus according to claim 5, wherein said optical sensing unit is adapted to sense individual photons, and wherein said apparatus is thereby adapted to sense light
25 emissions resulting from the stimulation of single molecules.

7. The apparatus according to claim 1, wherein the partition membrane is coated with at least one layer of a material having a high secondary electron yield.
8. The apparatus according to claim 1 wherein the partition membrane is adapted to
5 withstand a pressure gradient of up to one atmosphere.
9. The apparatus according to claim 9 wherein said membrane comprises a material selected from the group consisting of polyimide, polyamide, polyamide-imide, polyethylene, polypyrrole and additional conducting polymers, parlodion, collodion,
10 Kapton, FormVar, Vinylec, ButVar, Pioloform, silicon dioxide, silicon monoxide, and carbon.
10. The apparatus of claim 9 wherein the membrane is polyimide.
11. The apparatus according to claim 10, wherein said membrane is reinforced with
15 a reinforcing grid placed between said membrane and said vacuum.
12. The apparatus according to claim 9, wherein said membrane has a thickness lying within a range of substantially 200 - 5000Å.
- 20
13. The apparatus according to claim 1, wherein said partition membrane is placed across an aperture having a diameter substantially within the range of 0.1 to 4mm.
14. The apparatus according to claim 13, wherein said diameter is substantially
25 1mm.

15. The apparatus according to claim 1, wherein said partition membrane is transparent to energetic electrons.

5 16. The apparatus according to claim 15, wherein said partition membrane is transparent to electrons having energies in excess of 2keV.

17. The apparatus according to claim 16, wherein said partition membrane is transparent to electrons having energies in excess of 3keV.

10

18. The apparatus according to claim 1, wherein said partition membrane is coated with a layer of a material having a high secondary electron yield.

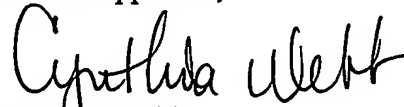
19. Use of an apparatus according to any one of claims 1-18 for detecting electron
15 beam fluorescence simultaneously with back scattered electrons.

20. Use according to claim 19 wherein sample is maintained in a wet environment.

21. Use according to claim 20 wherein the sample comprises biological cells.

20

For the applicant,



Cynthia Webb
Webb & Associates

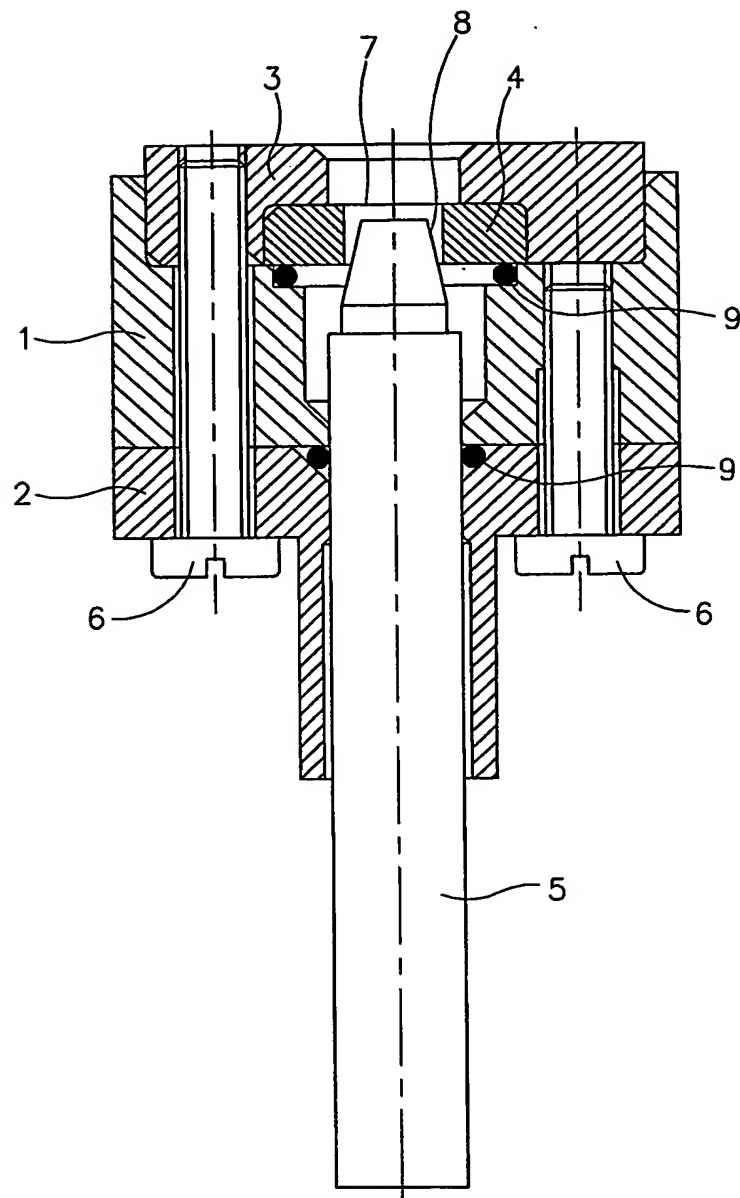


FIG.1A

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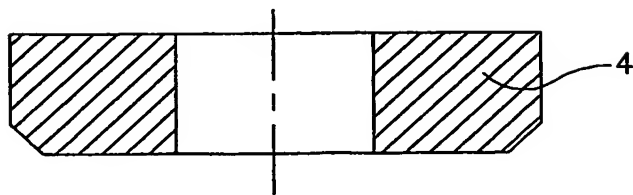


FIG.1B

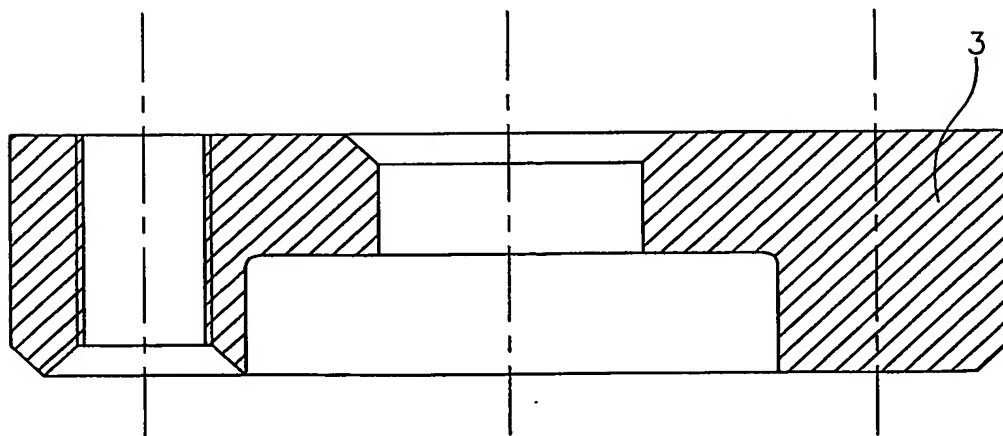


FIG.1C

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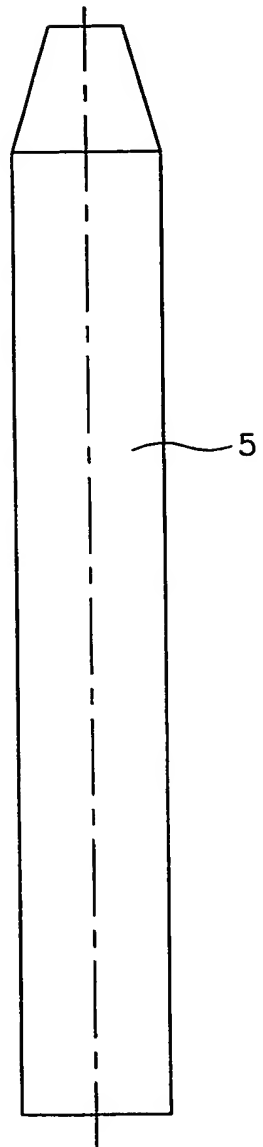


FIG.1D

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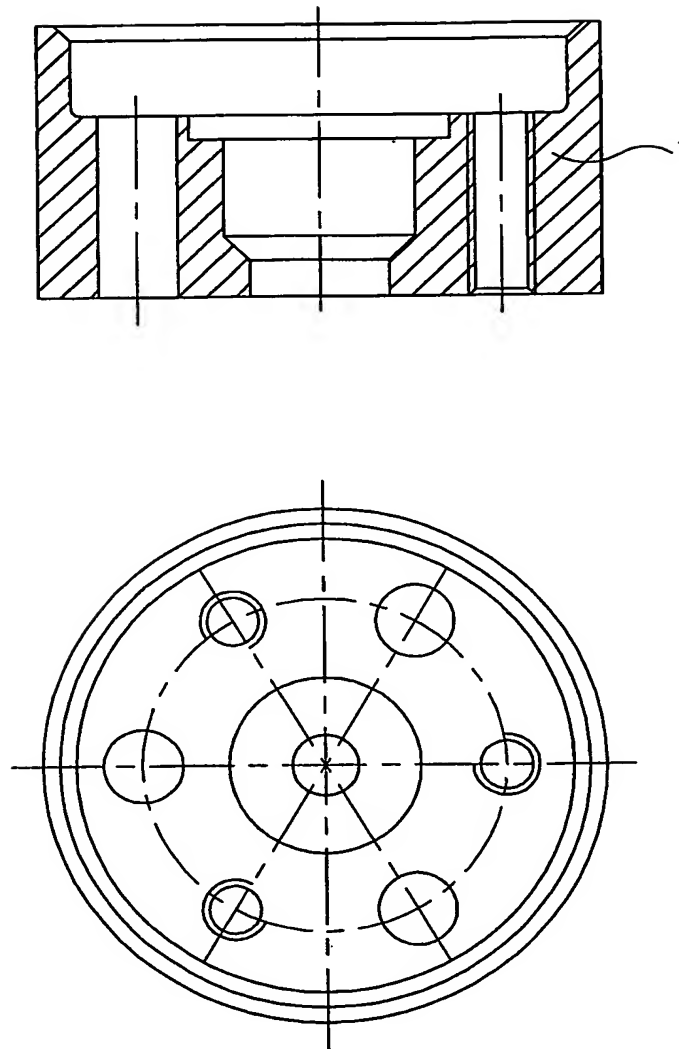


FIG.1E

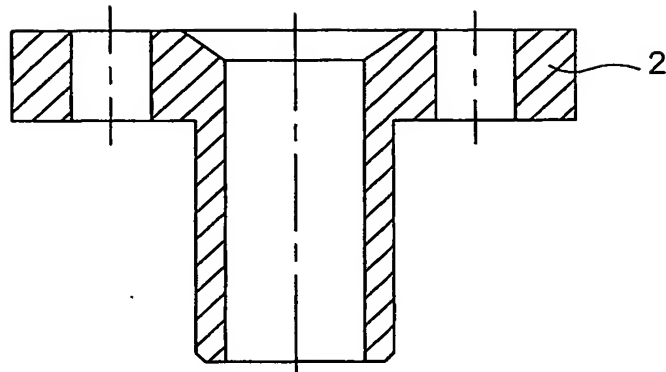


FIG.1F

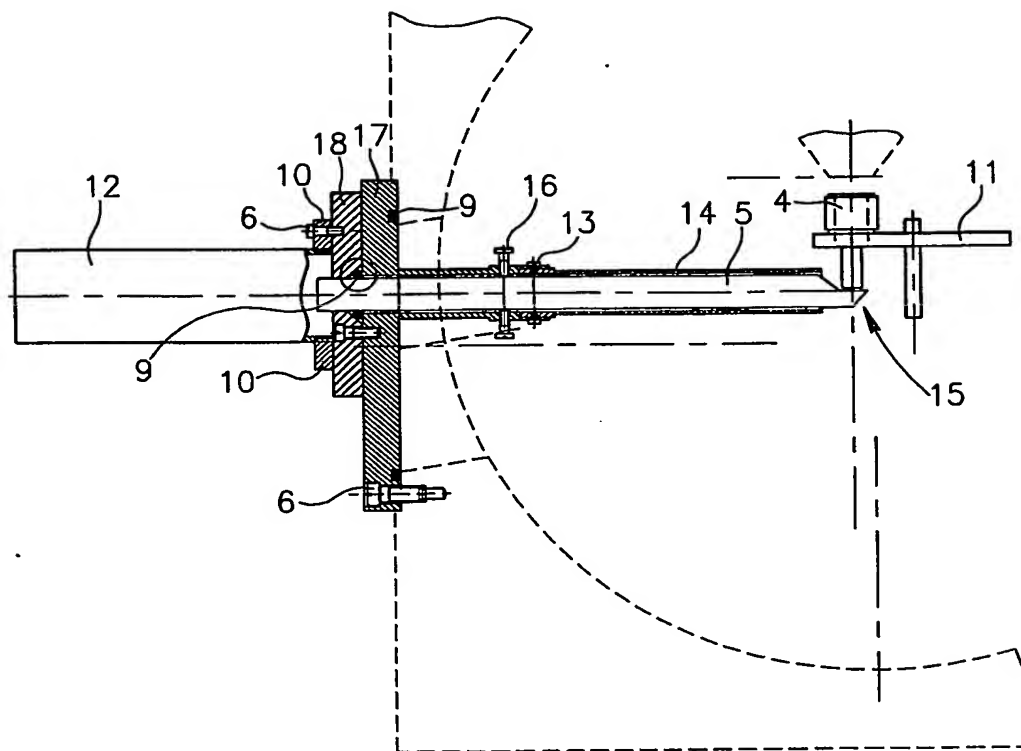


FIG.2A

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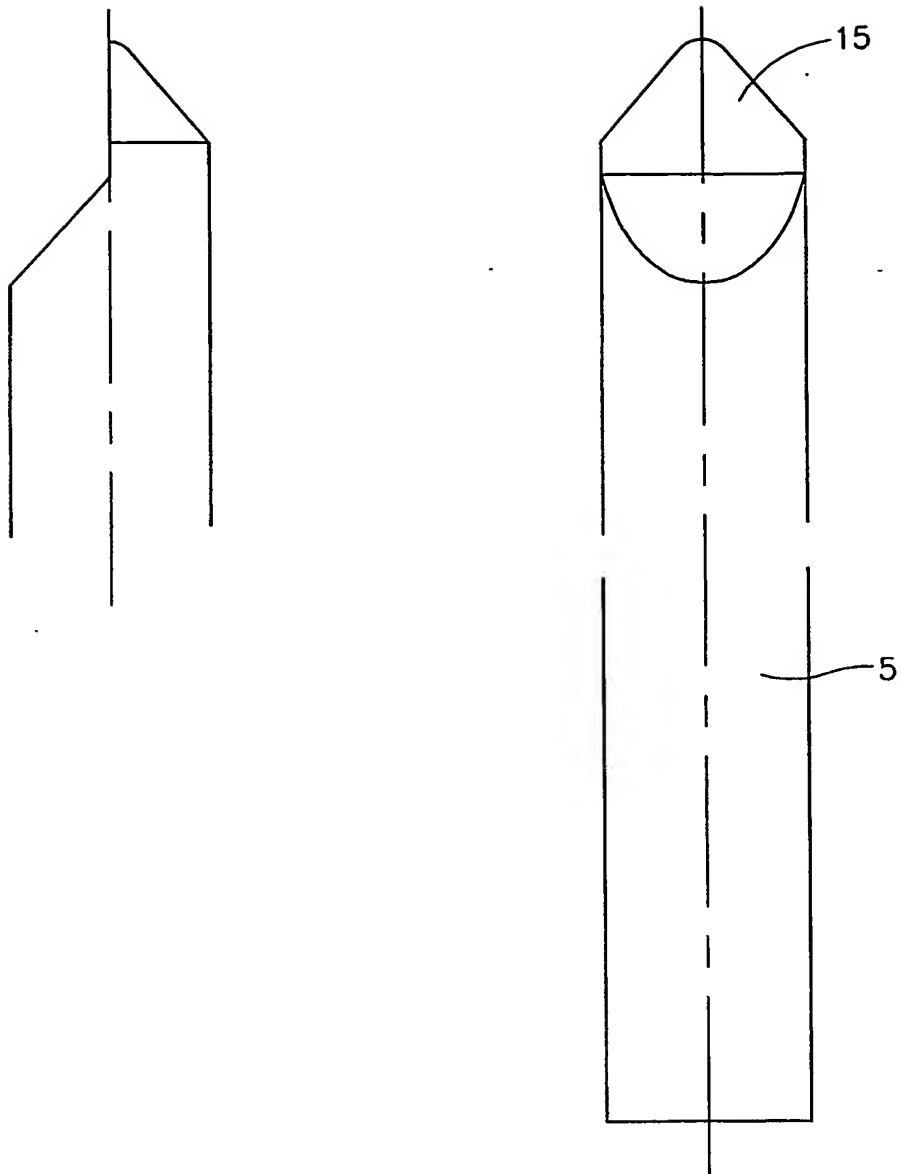


FIG.2B

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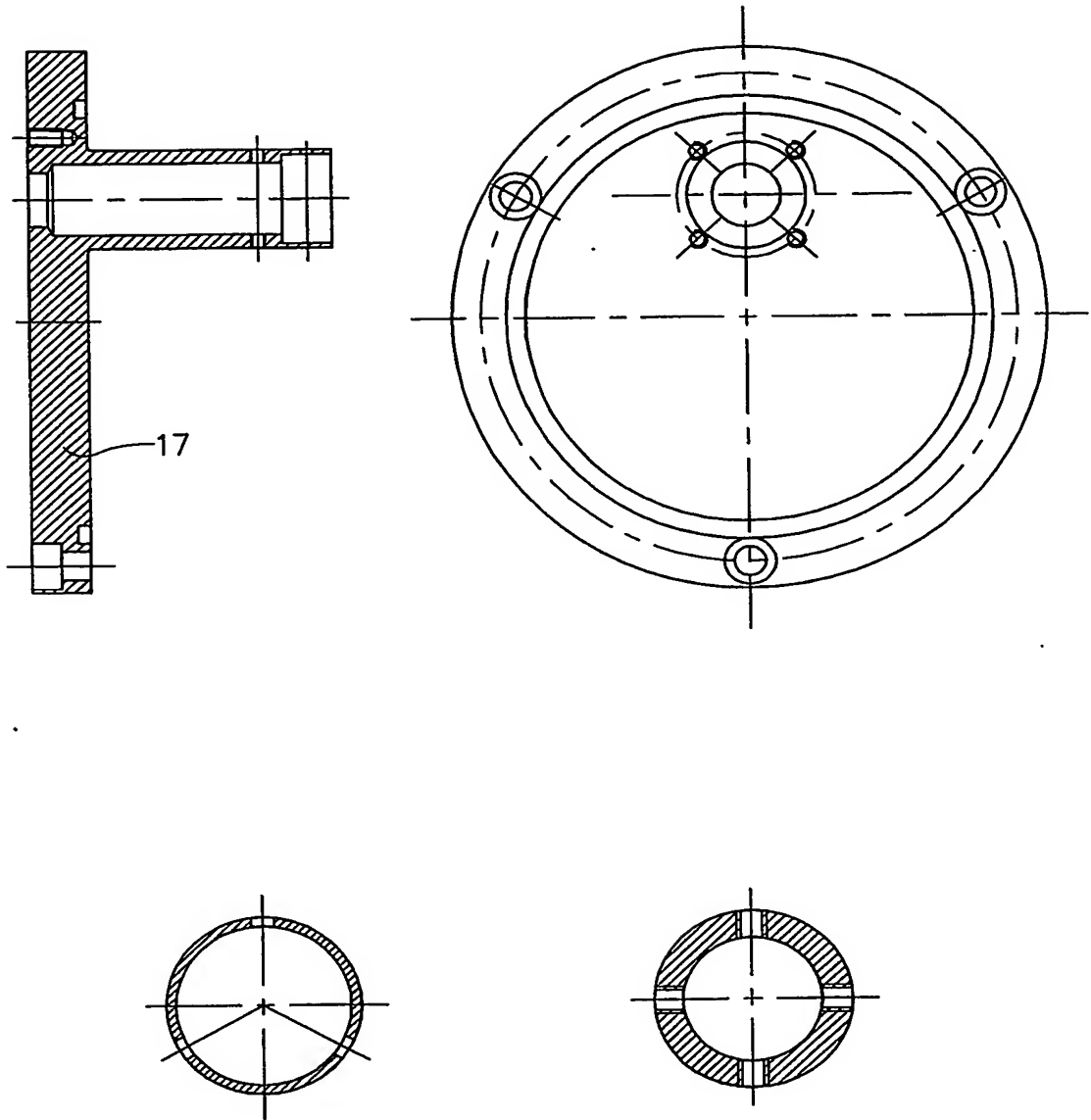


FIG. 2C

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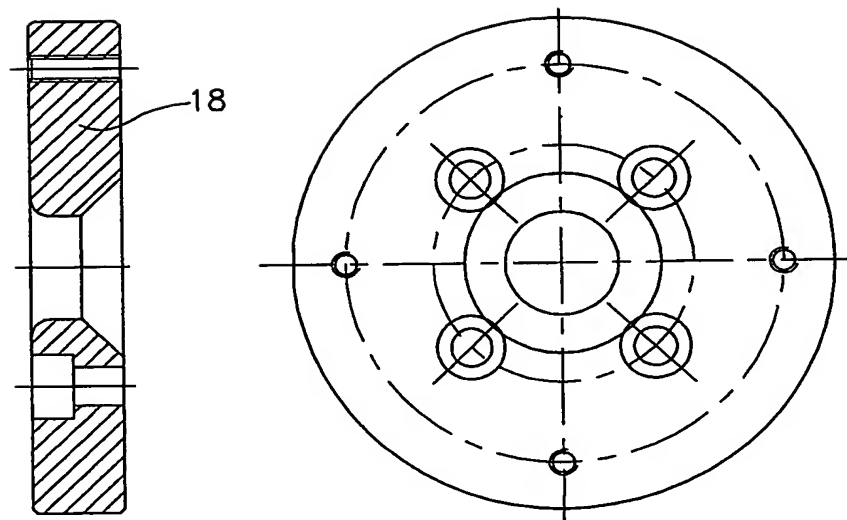


FIG.2D

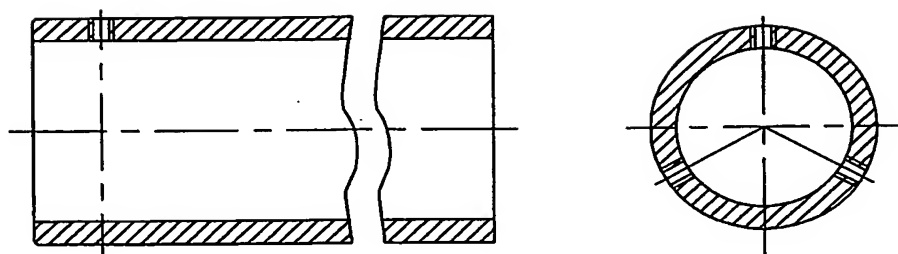


FIG.2E

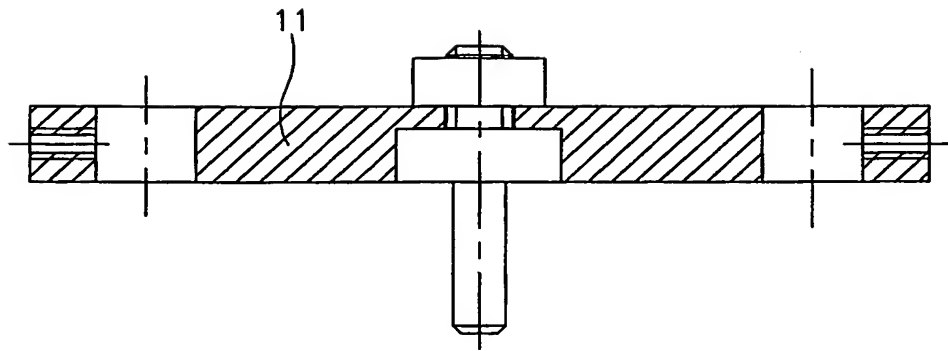


FIG.2F

Figure 3

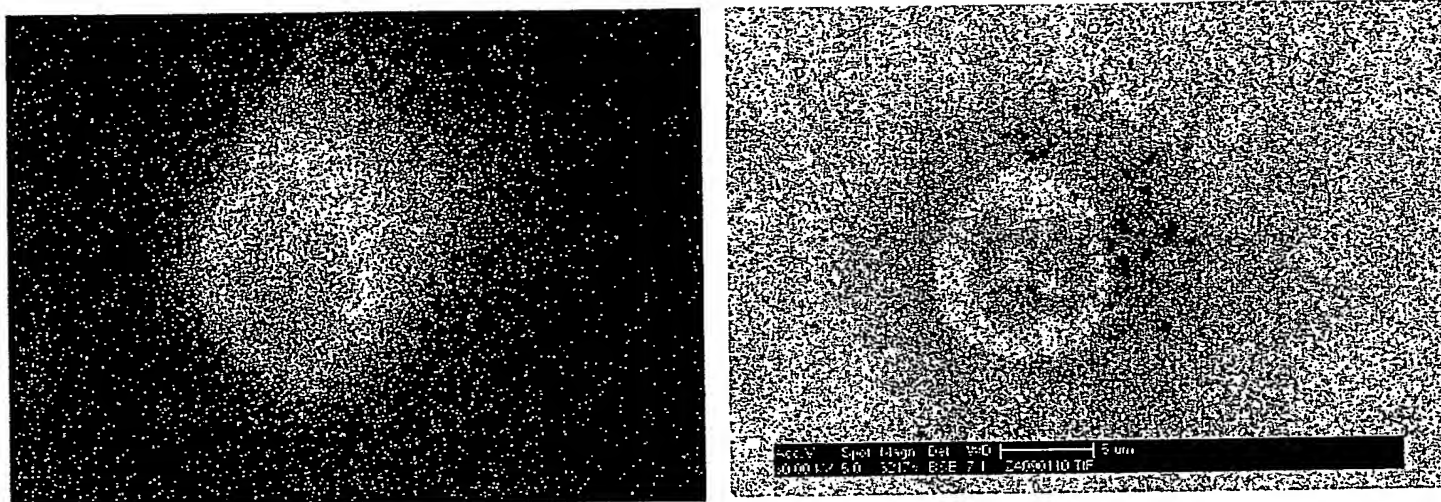
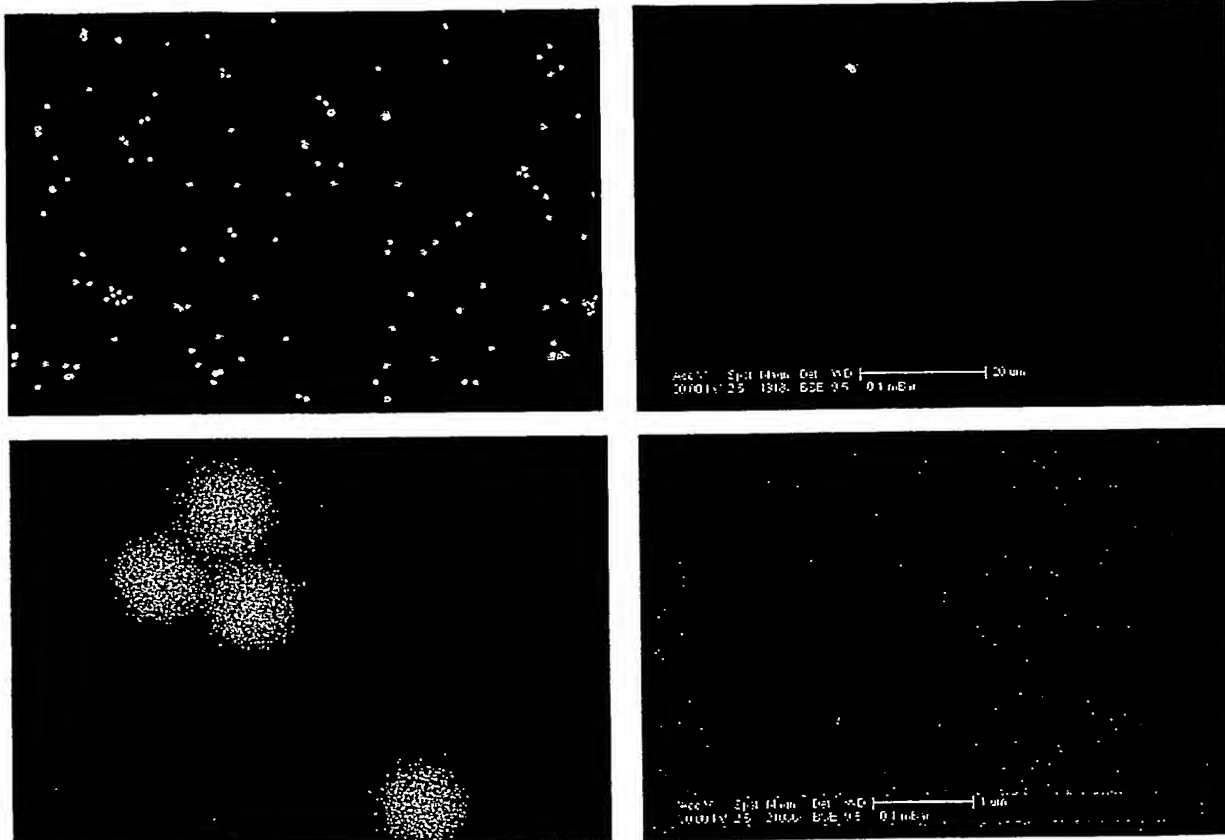


Figure 4



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